STUDIES ON PROTEINS OF ANIMAL RIBOSOMES. AFFINITY LABELING OF RAT LIVER RIBOSOMES BY *N*-BROMOACETYLPUROMYCIN*

J. STAHL, K. DRESSLER and H. BIELKA

Central Institute of Molecular Biology of the Academy of Sciences of GDR, Department of Cell Physiology, 1115 Berlin-Buch, GDR

Received 15 August 1974

1. Introduction

Recently *N*-iodoacetylpuromycin was shown to bind irreversibly to *E. coli* ribosomes [1] and to react preferentially with protein L6 and to lesser extent with protein L2 [2]. The puromycin derivative can be regarded as an analogue to the product of the puromycin reaction, *N*-peptidylpuromycin. Thus, it can be expected that the affinity label predominantly reacts with proteins in or very close to the peptidyltransferase centre at the puromycin binding site.

Another derivative of puromycin, 5′-*O*-(*N*-bromoacetyl-*p*-aminophenylphosphoryl)3′-*N*-L-phenylalanylpuromycin, however, when reacted in high excess with *E. coli* ribosomes binds almost exclusively to ribosomal RNA of the large subunit [3].

In this paper we show that a considerable part of *N*-bromoacetylpuromycin binds specifically to L27 and L29, two ribosomal proteins of the large subunit of rat liver ribosomes [4]. The binding efficiency of the affinity label to the ribosomes is strongly increased under conditions of high ionic strength (0.5 M KCl) known to support the puromycin catalyzed release of nascent proteins from polysomes [5]. The reaction of the affinity label is competitively inhibited by the presence of puromycin. From these results it is concluded that proteins L27 and L29 are located very close to the ribosomal peptidyltransferase centre at the ribosomal A-site.

* Part XVII of a Series.

2. Material and methods

The synthesis of *N*-bromoacetylpuromycin and *N*-bromo-[2-^{14}C]-acetylpuromycin, with a specific activity of 55 Ci/mole, is described by Pongs et al. [1]. We are grateful to Dr Olaf Pongs for the generous gift of the affinity label and instructive discussions.

Ribosomes were prepared from a postmitochondrial supernatant of rat liver by addition of Triton X-100 to a final concentration of 2% and centrifugation for 90 min at 105 000 *g*. The ribosomes were resuspended in TKM buffer (5 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 7.7).

Ribosomal subunits were prepared by puromycin-treatment basically according to Blobel and Sabatini [5].

Preparation of ribosomal protein and twodimensional polyacrylamide gel electrophoresis followed the method of Welfle et al. [4,6]. For determination of the radioactivity in the gel slabs the spots stained by Amido-black were cut out, incubated with 1 ml 0.5 M hyamine hydroxyde in toluene at 50°C for 16 hr in closed vials. After addition of 9 ml toluenebased scintillation fluid containing 10% Triton X-100 and 3% glacial acetic acid the samples were counted in a LKB–Wallac Liquid-Scintillation-Spectrometer to an error of 5%.

Incorporation of *N*-bromoacetylpuromycin into ribosomes was tested by incubation at 37°C for various periods under the conditions given in the legends. The reaction was stopped by adding TCA to a final concentration of 10%. The precipitates were filtered onto glasfibre filters, GFA (Whatman), dried and counted in toluene-based scintillation fluid.

3. Results and discussion

To find suitable conditions for the reaction of *N*-bromoacetylpuromycin with ribosomes the time course of this reaction was studied. On mixing equimolar amounts of radioactive affinity label with ribosomes mainly consisting of polysomes, the reaction was very fast and a plateau was reached after 10 min (fig. 1) but only 10% of the label is bound. The reactivity of the ribosomes is considerably improved and the maximal amount of affinity label bound is increased to a value of about 22% when using polysomes pretreated with puromycin.

Blobel and Sabatini [5] reported that the puromycin catalyzed release of nascent protein from liver polysomes is accelerated in the presence of 0.5 M KCl. Incubation under these conditions leaves ribosomes intact and also allows isolation of subunits active in protein synthesis. At 0.5 M KCl and 5 mM $MgCl_2$ the reactivity of *N*-bromoacetylpuromycin with ribosomes is considerably increased. To determine if *N*-bromoacetylpuromycin reacts specifically with puromycin treated ribosomes, competition experiments

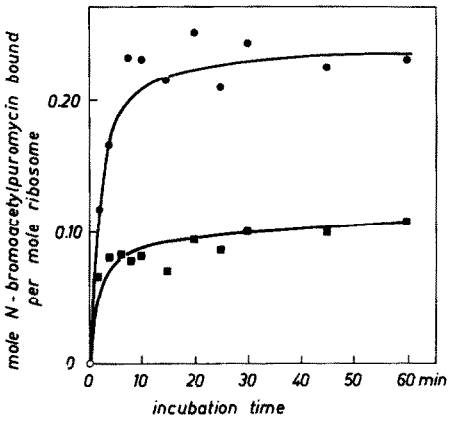

Fig. 1. Reaction of *N*-bromoacetylpuromycin with ribosomes. a) Polysomes (■——■) were incubated in a concentration of about 15 mg/ml with an equimolar amount of 3.3 nmoles of ^{14}C-*N*-bromoacetylpuromycin in TKM-buffer at 37°C. At various intervals samples of 100 µl each were taken and the incorporated activity measured. b) Polysomes, pretreated with puromycin (●——●) were prepared by addition of 200 moles puromycin per mole ribosome, incubation for 20 min at 0°C and 10 min at 37°C. Then the affinity label was added, the incubation continued and samples taken as described above.

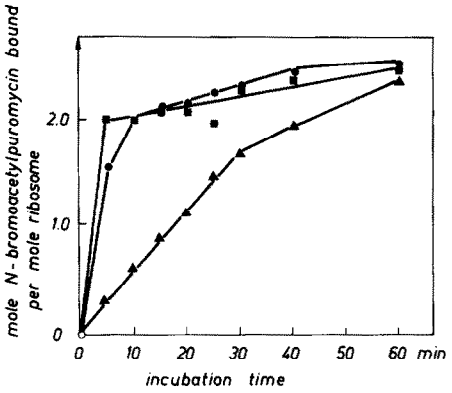

Fig. 2. Influence of puromycin on the binding of *N*-bromoacetylpuromycin to ribosomes. Ribosomes were preincubated with a 200-fold excess of puromycin for 20 min at 0°C and 10 min at 37°C, isolated by centrifugation through a cushion of TKM containing 0.4 M sucrose, for 2 hr 50 000 rpm, rotor 65, Spinco, and resuspended in TKM-buffer containing 0.5 M KCl. Then 25 pmoles of ribosomes were added to a reaction mixture containing 10 mM Tris–HCl, pH 7.7, 0.5 M KCl, 1.5 mM $MgCl_2$ and 125 pmoles *N*-bromoacetylpuromycin plus a) no puromycin (■——■), b) 2500 pmoles puromycin (●——●) and c) 25 000 pmoles of puromycin (▲——▲) (compare table 1) in a total vol of 0.400 ml each. Samples of 20 µl were taken and the TCA precipitable radioactivity determined.

with puromycin were performed. For this purpose ribosomes were preincubated with a 200-fold excess of puromycin for 20 min at 0°C and 10 min at 37°C to release most of the nascent protein. These particles were centrifuged through a cushion of 0.5 M sucrose in TKM-buffer to remove puromycin. To the ribosomes prepared in this way puromycin in two different concentrations and *N*-bromoacetylpuromycin in a constant amount were added in the cold and then incubated at 37°C. Samples were taken at various intervals as indicated in fig. 2. From this figure it is obvious that puromycin competes with *N*-bromoacetylpuromycin binding to the ribosome and inhibits the reaction considerably. In all cases, however, a maximal binding of about 2.0 moles *N*-bromoacetylpuromycin per mole ribosome is reached. As can be taken from table 1, the initial rate of the reaction is lowered by a factor of about 7, when applying a 200-fold excess of puromycin over *N*-bromoacetylpuromycin. These data point to the assumption that the affinity label binds to the same ribosomal site as puromycin and therefore reacts specifically with the ribosome.

Table 1
Influence of puromycin on the binding of *N*-bromoacetylpuromycin to ribosomes

Ribosomes (pmoles)	*N*-bromoacetyl-puromycin (pmoles)	Puromycin (pmoles)	Moles affinity label bound per mole 80 S × sec^{-1}
25	125	0	6.7×10^{-3}
25	125	2 500	5.0×10^{-3}
25	125	25 000	1.0×10^{-3}

Experimental conditions were as given in the legend to fig. 2. The numbers in the last column represent the initial velocities of the binding reaction.

In further experiments *N*-bromoacetylpuromycin was incubated with puromycin pretreated ribosomes in various proportions between 1:1 and 8:1. Fig. 3 shows, that the amount of affinity label bound to ribosomes increases when the proportion of *N*-acetyl-puromycin to ribosomes is increased. From these results it appears that the proportions used are still far too small to reach saturation of the system. At higher proportions the unspecific reaction of the bromo-moeity of the reagent with reactive groups outside the puromycin binding site is increased in parallel to an increase of the specific binding of the affinity label to proteins in the A-site.

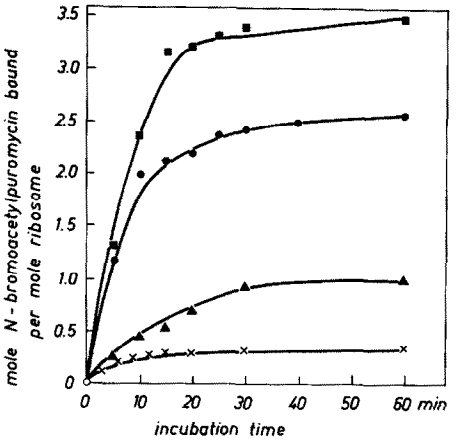

Fig. 3. Extent of binding of *N*-bromoacetylpuromycin to ribosomes at various proportions between both components. To ribosomes preincubated with a 200-fold excess of puromycin and 0.5 M KCl for 20 min at 0°C and 5 min at 37°C different amounts of affinity label were added. The molar relations between *N*-bromoacetylpuromycin and ribosomes were 1:1 (×——×), 2:1 (▲——▲), 5:1 (●——●) and 8:1 (■——■). Aliquots were taken after various intervals and TCA precipitable activity determined.

To determine which of the ribosomal proteins are preferably labeled by *N*-bromoacetylpuromycin the affinity label was used in a 5:1 to 10:1 excess with puromycin treated ribosomes. Then, the ribosomes were dissociated into subunits, their proteins isolated,

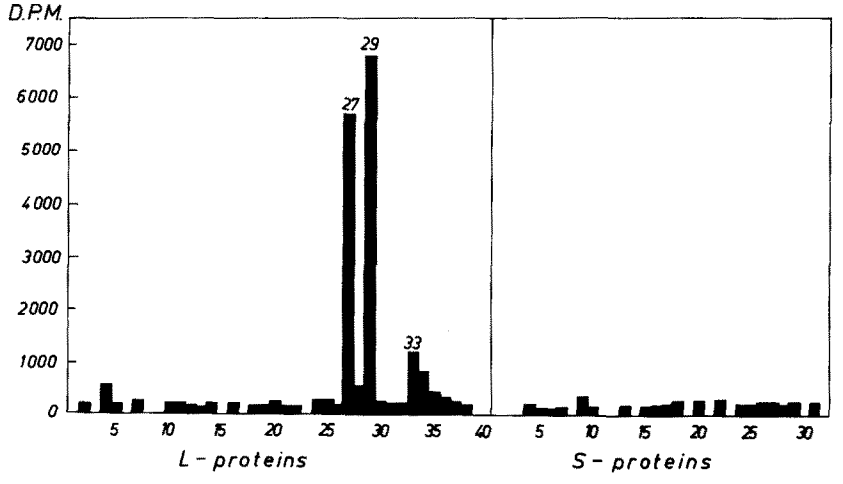

Fig. 4. Distribution pattern of ^{14}C-radioactivity in ribosomal proteins after separation by 2 D-electrophoresis.

separated by two dimensional gel electrophoresis and stained with Amido black. After destaining the spots were cut out and the bulk of radioactivity was found in two spots of the large ribosomal subunit, proteins L27 and L29, in almost identical amounts (fig. 4). From the Amido black staining intensity and the molecular weights of the two proteins it was roughly calculated that about 5–10% of L27 and L29 had reacted with the affinity label.

In addition to L27 and L29, in few experiments some radioactivity was found in protein L33. This labeling does not seem to be specific because L33 is rather reactive with other agents, as for instance iodoacetamide [7]. L27 and L29, however, do not react to a remarkable extent with iodoacetamide when organized in the ribosome structure.

References

[1] Pongs, O., Bald, R. and Erdmann, V. A. (1973) FEBS Letters 35, 137.
[2] Pongs, O. and Erdmann, V. A. (1974) Proc. Natl. Acad. Sci. U.S., in press.
[3] Harris, R. J., Greenwall, P. and Symons, R. H. (1973) Biochem. Biophys. Res. Commun. 55, 117.
[4] Welfle, H., Stahl, J. and Bielka, H. (1972) FEBS Letters 26, 228.
[5] Blobel, G. Sabatini, D. (1971) Proc. Natl. Acad. Sci. U.S. 68, 390.
[6] Welfle, H., Stahl, J. and Bielka, H. (1971) Biochim. Biophys. Acta 243, 416.
[7] Iwasaki, K., Mizumoto, K., Tanaka, M. and Kaziro, Y. (1973) J. Biochem. 74, 849.
[8] Westermann, P., Heumann, W. and Bielka, H. (1974) Acta Biol. Med. Germ. 33, in press.